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Infection of Human Endothelial Cells with *Bartonella bacilliformis* Is Dependent on Rho and Results in Activation of Rho

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***Bartonella bacilliformis* was continuously internalized into human endothelial cells beginning shortly after addition of the bacteria and continuing for at least 24 h after infection in vitro, with a major increase in uptake occurring between 16 and 24 h. Preincubation of endothelial cells with C3 exoenzyme, which inactivated intracellular Rho-GTPase, blocked internalization of the bacteria. Addition of C3 exoenzyme at any time after addition of the bacteria blocked further internalization of bacteria, including the major uptake of bacteria internalized at 16 to 24 h. Rho, a key signaling protein in pathways involving actin organization, was directly shown to be activated in endothelial cells undergoing infection with *B. bacilliformis*, with maximal activation and translocation to the plasma membrane at 12 to 16 h. At late times of infection, most of the bacteria were found in a perinuclear location. Staining of the Golgi complex with specific markers, anti-human Golgin-97, anti-KDEL receptor, and BODIPY-TR ceramide, showed colocalization of bacteria in the Golgi complex region. Disruption of the Golgi complex with brefeldin A scattered the bacteria from this perinuclear location and resulted in inhibition of internalization of the bacteria in endothelial cells.**

Bartonella bacilliformis is a gram-negative, facultative intracellular bacterium which enters the bloodstream of humans through the bite of a sand fly vector. During the initial hematic phase (Oroya fever), *B. bacilliformis* can parasitize up to nearly 100% of erythrocytes, leading to their premature destruction and to a severe, often fatal hemolytic anemia. In the nonfatal tissue phase of the disease (Carrion's disease), *B. bacilliformis* invades endothelial cells, causing the appearance of hemangioma-like papules or nodules, called verrugas, which can persist for more than a year. Several other pathogenic bacteria are also known to invade endothelial cells in vivo and in vitro (4, 12, 21, 34, 39).

Bartonella may enter epithelial or endothelial cells in vitro either as individual bacteria or in small or large clumps (3, 17). Initial adherence of *Bartonella henselae* is mediated by the expression of type IV-like pili (3, 27). Inhibition of internalization of *B. bacilliformis* by cytochalasin D suggests the active involvement of host cell and cytoskeleton rearrangement in the invasion process (17). Following adhesion to endothelial cells in vitro, *B. henselae* are transported by the leading lamella of the cell and then engulfed by fusion of membrane protrusions over a period of about 24 h (10). This process required a reorganization of the host cell cytoskeleton, which had also been observed in endothelial cells following infection by *B. henselae* and *Bartonella quintana* (29).

Intracellular pathogens exploit host cell signaling pathways to facilitate their uptake and survival within host cells. Some bacteria, such as *Salmonella* and *Shigella*, inject virulence factors by using Type III transport systems, which produce membrane ruffling and actin rearrangements at the cell surface that facilitate bacterial uptake. *Listeria*, *Yersinia*, and *Neisseria* organisms enter by binding to host cell receptors that function as part of the endocytosis system (for reviews, see references 13, 15, and 18). Remodeling of the mammalian cell surface, often involved in bacterial entry, can be accomplished through reorganization of the actin cytoskeleton, which is regulated in part by the small GTP-binding proteins of the Rho family (Rho, Rac, and Cdc42). Rho-GTPases function as molecular switches by cycling between an inactive state with bound GDP and an active state with bound GTP. The active form of Rho interacts with downstream effector proteins to produce biological responses, which include actin reorganization.

C3 exoenzyme, a bacterial toxin from *Clostridium botulinum*, ADP-ribosylates RhoA, RhoB, and RhoC (2) at Asn^{41} (in the putative effector region of Rho [33]), which blocks Rho-dependent signaling to its downstream effectors. This toxin, as well as a similar toxin from *Staphylococcus aureus*, inactivates Rho and induces actin depolymerization, which can lead to altered morphology and function (22).

Infection of endothelial cells with *B. bacilliformis* altered the morphology and cytoskeletal arrangement of the cell. Thick F-actin stress fibers arranged in parallel orientation along the long axis of the cell were formed, and they terminated in the increased number of focal contacts with the extracellular matrix. Cell motility was greatly decreased in these infected cells, and they were unable to participate in the formation of a capillary network in three-dimensional collagen gels (A. Verma, G. E. Davis, and G. M. Ihler, unpublished data). Stress fiber formation and motility is dependent on Rho-GTPase, suggesting that bacterial modification of Rho signaling pathways might be a feature of infection by *B. bacilliformis*. The results presented here directly demonstrate that activation of Rho is required for internalization of *B. bacilliformis* and that *B. bacilliformis* activates intracellular Rho in endothelial cells.

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MATERIALS AND METHODS

Bacterial strains. *B. bacilliformis* was routinely grown at 28°C in phosphate-buffered saline (PBS) over brain heart infusion (BHI) agar plates containing 10%

defibrinated sheep blood (Dickinson Microbiology System, Cockeysville, Md.) (5). *Escherichia coli* expressing the C3 exoenzyme gene from *C. botulinum* type D strain 1873 (30) cloned into the pGEX expression vector system (Pharmacia Biotech, Inc.) was kindly provided by Bradley McIntyre (University of Texas, M. D. Anderson Cancer Center, Houston, Tex.). pGEX DNA containing the Rho-binding domain (RBD) of Rhotekin (pGEX-RBD) was kindly provided by Martin A. Schwartz (The Scripps Research Institute, La Jolla, Calif.) and was transformed into *E. coli* DH5α

Antibodies and reagents. Mouse monoclonal antibodies directed against RhoA was obtained from Transduction Laboratories (a Becton Dickinson company; Los Angeles, Calif.). Mouse monoclonal antibody to platelet/endothelial cell adhesion molecule-1 (PECAM-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Research Diagnostics (Flanders, N.J.). Rabbit anti-mouse horseradish peroxidase conjugate and rabbit anti-mouse immunoglobulin G-tetramethyl rhodamine isocyanate (IgG-TRITC) were obtained from DAKO. DAPI (4′,6′-diamidino-2-phenylindole), mouse monoclonal antibody against human Golgin 97 (a specific marker for Golgi complex), and BODIPY-TR ceramide (*N*-(4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy) acetyl) sphingosine were from Molecular Probes. Mouse monoclonal antibody against the KDEL receptor was from Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada. ECL detection kit and alpha-thrombin was purchased from Pharmacia. Phenylmethylsulfonyl fluoride (PMSF), benzamidine hydrochloride, thymidine, and brefeldin A (BFA) were purchased from Sigma.

Transfer of pCD353 into *B. bacilliformis* by conjugation. Conjugation to generate fluorescent bacteria was performed essentially as previously described (9). Briefly, three parental matings were performed. Plasmid pCD353 from *E. coli* was transferred to *B. bacilliformis* with the helper plasmid pRK2013. *E. coli* β 2150 strains carrying expression vector pCD353 (containing green fluorescent protein [GFP] gene as an expression marker) and helper plasmid pRK2013 were grown overnight in 10 ml of Luria-Bertani (LB) medium containing kanamycin (30 μg/ml) and 1 mM diaminopimelic acid at 37°C with shaking. *E. coli* cultures were harvested and cells were resuspended in 100 μl of PBS, and a 2-day-old culture of *B. bacilliformis* was also harvested and resuspended in 100 μl of PBS. After mixing, the bacteria were dotted on nitrocellulose discs on a BHI blood agar plate overlaid with diaminopimelic acid and incubated for 8 h at 30°C. After conjugation, bacterial cells were collected from nitrocellulose discs by scrapping and washing the discs with PBS. The suspension was plated on the BHI blood agar plates overlaid with kanamycin (60 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (250 μM). Plates were incubated for at least 7 to 10 days at 28°C to obtain transconjugates.

Infection of human endothelial cells with *B. bacilliformis*. Human umbilical vein endothelial cells (Clonetics Corp., San Diego, Calif.) were propagated between passages 2 and 7 as previously described (8). Human umbilical vein endothelial cells were seeded on glass coverslips, in tissue culture flasks, and in six-well plates coated with gelatin (1 mg/ml), in M199 medium containing 20% fetal bovine serum, heparin, and bovine hypothalamic extract (26) for infection and/or immunofluorescent staining. For infection, transconjugated fluorescent *B. bacilliformis* organisms from a 2-day-old culture induced with 250 μM IPTG were used throughout the study (unless otherwise indicated). Cultures were harvested and washed several times with PBS and resuspended in plain M199. The cells were infected with 100 bacteria per cell. Infected monolayers were maintained at 37°C with 5% CO_2.

Flow cytometric analysis. For flow cytometric analysis, infected monolayers were washed five times with PBS. Removal of the extracellular adherent bacteria could be achieved by trypsin treatment as described earlier for *Bartonella* (17) and *Borrelia* (25). Briefly, trypsinized cells were centrifuged at 200 × *g* to avoid pelleting of remaining free-floating extracellular bacteria and the cell pellets were washed again two times with PBS. To distinguish between the extracellular and intracellular bacteria, trypsinized infected endothelial cells were counterstained with ethidium bromide (EB) (50 μg/ml) for 5 min as previously described (14). Cells were again washed two times with PBS and finally resuspended in PBS at a concentration of 10^6 cells/ml and observed with a fluorescent microscope. A total number of 10,000 counterstained infected endothelial cells were analyzed by flow cytometry, simultaneously for green and red emissions (28). Data were analyzed to obtain mean fluorescence and the percentage of fluorescing cells, using corresponding uninfected cells as controls.

Immunofluorescence staining of endothelial cells. Infection was terminated at selected time points by several washes of PBS, and subsequently cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were washed several times with PBS, postfixation. Uninfected and infected monolayers on glass coverslips were blocked with two washes of Tris-glycine buffer, and the cells were permeabilized with 0.5% Triton X-100 for 15 min. Coverslips were then blocked overnight at 4°C in PBS containing 0.1% Triton X-100, 1% bovine serum albumin (BSA), 1% normal rabbit serum, and 0.2% sodium azide. After overnight incubation, primary antibody was added to the same solution and incubated for 1 h at room temperature. Coverslips were washed three times with PBS and further incubated with secondary antibody (rabbit anti-mouse IgG-TRITC). Coverslips were again washed with PBS and mounted on the slides by using a fluorescent mounting medium (DAKO), and fluorescent images were captured in the Axiophot II digital imaging system in the Imaging Analysis Lab. The brightness and contrast of each image were identically modified in Adobe Photoshop 5.0. For confocal microscopy with cells fixed on glass coverslips, the scans were performed at 0.5-μm intervals in the *z* dimension beginning at the bottom of the cell (coverslip) and progressing up through the nucleus and over the top of the cell. Three-dimensional reconstructions of images were performed following *z* sectioning.

BFA treatment and staining of Golgi complex. Confluent monolayers of uninfected and infected endothelial cells on coverslips were fixed with 4% paraformaldehyde and washed several times with Hanks balanced salt solution (HBSS)-HEPES buffer or PBS. The cells were stained with antibodies to various markers of the Golgi complex, anti-human Golgin-97 and anti-KDEL receptor as described above, and with the BODIPY-TR ceramide. Coverslips were incubated with 5 μM BODIPY-TR ceramide coupled with defatted BSA at 4°C for 30 min. Coverslips were washed several times with the HBSS-HEPES buffer containing defatted BSA to remove the excess of ceramide, followed by washing with HBSS-HEPES, and mounted. Endothelial cells were treated with BFA prior to infection to disrupt the Golgi complex at a final concentration of 1 μg/ml, and BFA was present throughout the infection period. After infection, cells were trypsinized and analyzed by flow cytometry to assess the effect of BFA treatment on internalization of bacteria.

C3 exoenzyme purification. The gene for C3 exoenzyme from *C. botulinum* type D strain 1873 (30) was cloned into the pGEX expression vector system (Pharmacia Biotech Inc.) to generate a glutathione *S*-transferase (GST) fusion (pGEX2T-C3) (11) protein. C3 exoenzyme was purified as previously described (37). Briefly, a single colony of *E. coli* (strain JM109) transformed with pGEX2T-C3 DNA was inoculated into 200 ml of LB medium containing 100 μg of ampicillin/ml and grown overnight at 37°C on a shaker. This culture was then added to 1,800 ml of LB medium containing ampicillin, shaken at 37°C for 1 h, and induced with IPTG (100 μg/ml), and the culture was grown at 37°C on a shaker for an additional 7 h. The bacteria were pelleted at 5,000 × *g*, and the cells were lysed in 80 ml of ice-cold PBS containing 1 mg of lysozyme/ml, 1% Triton X-100, 25% sucrose, 1 mM EDTA, 5 mM β-mercaptoethanol, and 1 mM PMSF for 30 min on ice with occasional shaking. The slurry was then passed through a French press and pancreatic DNase I (Sigma) was added to a final concentration of 100 μg/ml, and then it was stirred for an additional 20 min at 4°C and centrifuged at 10,000 × *g* for 10 min at 4°C.

The lysate was added to 2 ml of washed glutathione-agarose beads (Pharmacia) and mixed gently at 4°C for 1 h. The beads were centrifuged at 500 × *g* for 5 min, washed four times with 50 ml of PBS containing 1% Triton X-100, and washed three times with 25 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2.5 mM $CaCl_2$, and the beads were resuspended in 1 ml of Tris buffer. Purified alpha-thrombin (60 NIH units) was added and incubated for 16 h at 4°C to cleave the fusion protein. The beads were centrifuged at 1,000 × *g* for 2 min, washed twice with 1 ml of the Tris solution, and concentrated using a Centricon-10 filter (Amicon, Beverly, Mass.). The product ran as a single band at approximately 25 kDa on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel.

In vivo ADP ribosylation of Rho in endothelial cells using C3 exoenzyme. Endothelial cells were subcultured in six-well culture dishes 24 h prior to the in vivo ADP ribosylation of Rho. To determine the concentration required for in vivo inactivation of Rho, endothelial cells were treated with 10, 20, 50, and 100 μg of C3 exoenzyme/ml, and the extent of ADP ribosylation of Rho in vivo in endothelial cells was determined by subsequent in vitro [^{32}P]ADP ribosylation. A concentration of 100 μg of C3 exoenzyme/ml was selected for the study. Cells were incubated with 100 μg of C3 exoenzyme/ml for 4, 8, or 12 h prior to infection and 0 (simultaneous addition of bacteria and C3 exoenzyme), 4, 8, 12, or 16 h after infection with *B. bacilliformis*.

In vitro [^{32}P]ADP ribosylation of Rho. Endothelial cell lysates were washed five times with ice-cold PBS, and the cells were lysed and sonicated in buffer containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM benzamidine hydrochloride, and 0.5 mM PMSF. About 10 μg of protein were added to a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 5 mM $MgCl_2$, 5 mM dithiothreitol, 2×10^6 cpm of [^{32}P]NAD (ICN), and 1 μg of C3 exoenzyme/ml in a final volume of 50 μl. The reaction mixture was incubated at 30°C for 1 h. The reaction was stopped by 5× Laemmli buffer, and proteins were electrophoresed in an SDS–18% polyacrylamide gel. The gel was stained with sypro orange (Bio-Rad) to confirm equal loading of protein and was autoradiographed.

Purification of GST-RBD and affinity precipitation of cellular GTP-Rho. pGEX2T-RBD construct DNA was transformed in *E. coli* DH5α and purified as described by Ren et al. (31). Briefly, protein expression was induced with 0.5 mM IPTG, and the cultures were lysed with a French press and the cleared lysate was mixed with washed glutathione beads. The beads were then washed and finally resuspended. Affinity precipitation of cellular-activated Rho (GTP-Rho) was performed. Briefly, endothelial cells infected with *B. bacilliformis* for different time intervals were scraped and resuspended in lysis buffer containing 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM orthovandate, 1 mM PMSF, 1% Triton X-100, 0.5% NP-40, 50 mM NaF, 10 μg of leupeptin/ml, and 1 μg of pepstatin/ml for 30 min at 4°C. Lysates were clarified by centrifugation at 16,000 × *g*, and protein was estimated (6). Equal amounts (300 μg) of the protein from cells infected for different times and uninfected cells were incubated for 1 h at 4°C with 50 μg of GST-RBD beads to capture activated Rho. The beads were then washed four times with wash buffer (50 mM Tris [pH 7.5], 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg of leupeptin/ml, 10

μg of aprotinin/ml, and 1 mM PMSF). GTP-bound Rho was eluted by boiling in 1× Laemmli sample buffer. The eluted protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto the polyvinylidine difluoride membrane, blotted with the anti-RhoA mouse monoclonal antibodies, and detected with the secondary antibody conjugated with horseradish peroxidase by using enhanced chemiluminescence, and for quantitative analysis, films were scanned by densitometry using an Alphaimager 2000 (Alpha Innotech, San Leandro, Calif.). To determine levels of total Rho in cell lysates prepared at different time intervals of infection, equal amounts of protein were loaded on SDS-PAGE gels and analyzed by Western blotting using anti-RhoA antibodies. To confirm the equal loading of the proteins, the same membrane was stripped and then blotted with the anti-GAPDH monoclonal antibodies.

Subcellular fractionation. Confluent monolayers of uninfected as well as infected endothelial cells (2 and 12 h) were washed with ice-cold PBS five times, and the cells were scraped into 0.5 ml of extraction buffer (50 mM β-glycerophosphate [pH 7.3], 1 mM EGTA, 1 mM orthovandate, 10 μg of leupeptin/ml, 0.15 U of aprotinin/ml, and 1 mM PMSF). Cell extracts were sonicated for 5 s on ice (setting of 40 W, frequency of 3.25 kHz; Branson B12 sonifier) and centrifuged at 250 × *g* to remove the unlysed cells. Cell lysates were further fractionated into particulate and cytosolic fractions at 100,000 × *g* for 1 h. The particulate pellet was resuspended in a volume of extraction buffer equal to that of the cytosolic fraction and further sonicated. Equal volumes of particulate and cytosolic fractions, corresponding to 800 μg of the protein of the total lysate, were used with GST-RBD affinity beads to capture GTP-Rho. Bound activated Rho was analyzed by Western blotting using anti-RhoA antibodies.

RESULTS

pCD353 carrying the GFP gene as an expression marker was introduced into *B. bacilliformis* to permit the course of infection to be monitored using fluorescence by either fluorescence microscopy or flow cytometry. Adherent, noninternalized bacteria could be effectively removed by trypsinization. To distinguish between extracellular and intracellular bacteria, infected endothelial cells were counterstained with EB for 5 min. Basically, intracellular GFP-expressing bacteria resist staining with EB and remain green, but extracellular adherent bacteria are stained with EB and appear orange by fluorescence microscopy. Prior to trypsinization, most of the extracellular bacteria associated with infected endothelial cells appeared orange and were present in large clumps. After trypsinization, the bacterial clumps became disaggregated and very few individual adherent extracellular bacteria were seen (0 to 5 orange bacteria attached to the infected endothelial cells containing the green bacteria). Flow cytometric analysis of the EB-counterstained, infected endothelial cells, monitored for red as well as green fluorescence emissions, confirmed and further quantitated the results obtained from fluorescence microscopy. Individual plots of the green and red emissions and typical dual dot plots of the uninfected cells (Fig. 1A) and infected endothelial cells without (Fig. 1B) and with (Fig. 1C) trypsinization are presented. Without trypsinization, about 90% of the endothelial cells were positive for red fluorescence emission, indicating the presence of extracellular adherent bacteria. After trypsinization, only 1 to 2% of the endothelial cells had EB (red) fluorescence emission, indicating the efficiency of the trypsinization for the removal of the adherent bacteria.

Intracellular localization of *B. bacilliformis* in infected endothelial cells. Using confocal microscopy, the fluorescent bacteria were located relative to nuclei visualized with DAPI staining. Optical sections (0.5 μm thick) taken through the entire thickness of infected endothelial cells revealed intracellular bacterial clumps, mostly localized to the perinuclear region (Fig. 2) and in the same planes as the nucleus. Using fluorescence microscopy, those bacteria which were scattered through the cytoplasm colocalized with the cell surface marker PECAM-1, which also intensively stained the cell-cell junctions. Later in infection, after the loss of the cell-cell junctions, the bacteria accumulated in the perinuclear region, which by this time stained more intensively with PECAM-1 (Fig. 3A). Infected cells were stained with several Golgi complex-specific markers, KDEL receptor (a 23-kDa integral membrane protein localized to the *cis*-Golgi and intermediate compartments that is responsible for the retrieval of soluble endoplasmic reticulum luminal proteins bearing the tetrapeptide KDEL [24, 36]), human Golgin 97 protein (a 97-kDa integral membrane protein localized on the cytoplasmic face of the Golgi apparatus which is a member of granin family of proteins [21]), and BODIPY-TR ceramide. When the individual images of Golgi complex and internalized fluorescent bacteria were merged, the bacteria were clearly localized in and around Golgi complex region (Fig. 3B, C, and D). When the Golgi complex was disrupted with BFA prior to infection, very few internalized bacteria were seen in the perinuclear area; rather, they were much more scattered in the cytoplasm (Fig. 3E). Flow cytometric analysis after 24 h of infection with BFA-treated endothelial cells showed a large decrease (>50%) in internalized bacteria (Fig. 3F), indicating that BFA treatment reduces bacterial internalization.

Continuous internalization of *B. bacilliformis* in endothelial cells during infection. Flow cytometric analysis of endothelial cells infected with *B. bacilliformis* for different time intervals showed that 25 to 30% of the endothelial cells were infected by 2 h, and the percentage progressively increased to 65% at 24 h (Fig. 4A), showing that infection of previously uninfected endothelial cells continued for at least 24 h. The initial rate of infection appeared to be substantially greater than the rate of infection observed at later times.

A progressive increase in the mean fluorescence of endothelial cells was observed (Fig. 4A), indicating that the number of bacteria per endothelial cell increased during the incubation. This increase could be due either to multiplication of intracellular bacteria or to continued internalization of extracellular bacteria (which continue to multiply extracellularly during the incubation), or perhaps due to both. A very marked increase in mean fluorescence was reliably observed between 16 and 24 h of infection. To further study internalization, endothelial cells were infected with nonfluorescent bacteria for 20, 16, or 12 h, followed by incubation with fluorescent bacteria for a total of 24 h. A large uptake of fluorescent bacteria and a much higher mean fluorescence was observed for the three cultures incubated without fluorescent bacteria for 20, 16, or 12 h and then with fluorescent bacteria for 4, 8, or 12 h (Fig. 4B), compared to those of their respective control cells infected with fluorescent bacteria for 4, 8, and 12 h only. This demonstrates that the bacteria are continuously internalized during the incubation and that the increase in fluorescence seen between 16 and 24 h is due to a much higher level of internalization of bacteria.

C3 exoenzyme pretreatment of endothelial cells inhibits internalization of *B. bacilliformis*. In preliminary experiments, the concentration of C3 exoenzyme and the time of incubation required to inactivate intracellular endothelial cell Rho were determined. In vitro ADP ribosylation of Rho in endothelial cell lysates, using ^{32}P-NAD and C3 exoenzyme, measured levels of Rho escaping C3 exoenzyme inactivation in vivo. A concentration of 100 μg of C3 exoenzyme/ml was found to be optimal to markedly inactivate Rho in endothelial cells, and this concentration was utilized throughout the study. Marked reduction of the levels of in vitro ADP ribosylation of Rho was observed after 8 or 12 h of incubation of endothelial cells with C3 exoenzyme, but not after 4 h of incubation (Fig. 5A). This indicates that there is a 4-h lag period before the effect of C3 exoenzyme on Rho activity can be observed.

To determine whether inactivation of Rho in endothelial cells affects *B. bacilliformis* internalization, C3 exoenzyme was added to endothelial cells at different times before and after infection. Endothelial cells were pretreated with C3 exoen-

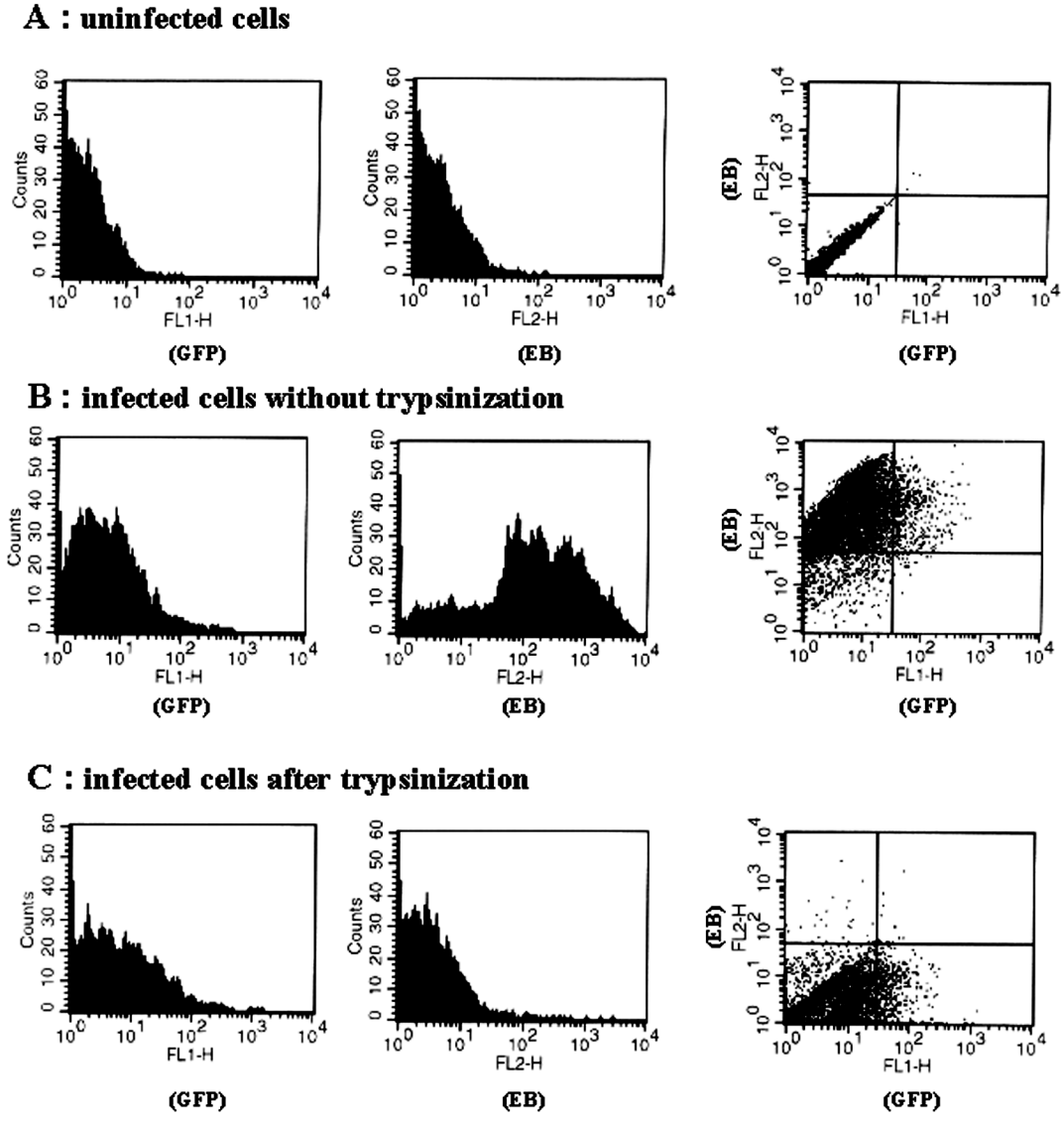


FIG. 1. Representative flow cytometric plots and dot plots of the uninfected and infected endothelial cells. (A) Flow cytometric plot of green and red fluorescence emissions and dual dot plot of uninfected endothelial cells. (B and C) Flow cytometric plots of the green and red fluorescence emission and dual dot plots of infected endothelial cells before and after trypsinization. Untrypsinized and trypsinized infected endothelial cells were counterstained with EB for 5 min (which can specifically stain extracellular bacteria) and analyzed simultaneously for green and red fluorescence emissions by flow cytometry. Dot plots show the distribution of endothelial cells having red and green fluorescence. In all the dot plots, the upper right quadrant contains the endothelial cells with intracellular green and extracellular adherent red bacteria, the lower right quadrant contains endothelial cells with intracellular green bacteria only, the upper left quadrant contains endothelial cells with adherent red bacteria only, and the lower left quadrant contains the endothelial cells with background minimal fluorescence emission.

zyme for 4, 8, or 12 h, followed by incubation with bacteria for an additional 24 h. Flow cytometry analysis showed that only a low percentage (not more than 4 to 8%) of endothelial cells preincubated with C3 exoenzyme became infected (Fig. 5B) during a subsequent 24-h incubation with bacteria, whereas 65% of endothelial cells not preincubated with C3 exoenzyme were infected.

When C3 exoenzyme was added at the same time as the bacteria or added at 4, 8, 12, or 16 h after initiation of infection, no substantial decrease in the percentage of infected cells was observed (Fig. 5C). This is consistent with the observation that at least 4 h of incubation with C3 exoenzyme is required to inactivate intracellular Rho and that 4 h is sufficient for infection of well over 50% of the cells that ultimately become infected within 24 h (Fig. 4A). However, when endothelial cells were incubated with C3 exoenzyme after initiation of infection, the marked increase in mean fluorescence intensity routinely observed at 16 to 24 h after infection was eliminated (Fig. 5D).

Rho is activated in infected endothelial cells and is translocated to the plasma membrane. Ren et al. (31) had developed an affinity capture system to precipitate the activated form of Rho (Rho-GTP) by using the Rho effector protein, Rhotekin, that interacts only with the GTP-bound form of Rho. This affinity precipitation system was used to determine whether infection of endothelial cells with *B. bacilliformis* leads to Rho activation or a change in its subcellular distribution, since activated Rho is translocated from the cytosol to the plasma membrane.

Extracts of infected or uninfected cells were incubated with beads bearing recombinant Rhotekin. After elution of the pro-

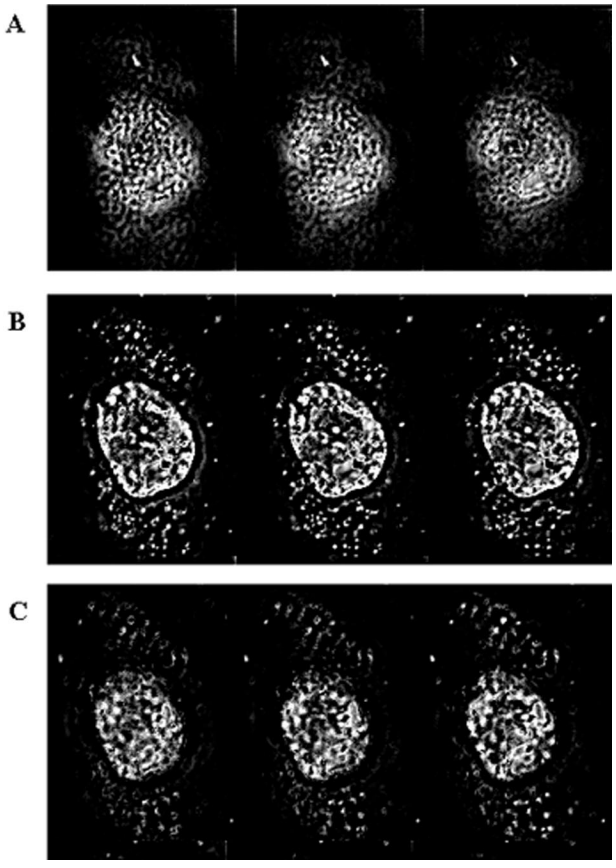


FIG. 2. Intracellular location of *B. bacilliformis* in infected endothelial cells. Confluent monolayers of endothelial cells were infected at a multiplicity of 1:100 with *B. bacilliformis* expressing GFP for 24 h, fixed with 4% paraformaldehyde, stained with nuclear stain DAPI, and analyzed by series of optical sectioning (0.5-μm-thick sections) through the entire thickness of the cell with confocal microscopy. Selected optical sections from the top (A) through middle (B) to the bottom (C) of the cell are shown. Note the focusing of the nucleus and the bacteria (B) in the perinuclear region in the same sections, confirming their intracellular presence.

tein from the beads, the relative amounts of activated Rho were determined using gel electrophoresis and Western blotting with anti-RhoA antibody. The amount of activated Rho increased with the time of the infection and reached a maximum between 12 and 24 h after the initiation of infection (Fig. 6A). Levels of total Rho in lysates from uninfected or infected cells were found to decrease slightly as the infection proceeded (Fig. 6B), although levels of a control protein (GAPDH) were constant over the time course of infection (Fig. 6C). Densitometric analysis of Rho-GTP levels relative to total Rho showed a threefold increase at 16 h of infection. At 24 h of infection, there appeared to be less activated Rho than at 16 h (Fig. 6D), but the levels relative to total Rho were still high, about 2.8-fold greater than those of the uninfected cells.

To determine the distribution of activated Rho between membrane and cytosol, cell lysates were fractionated by centrifugation at 100,000 × *g* for 1 h using control and infected endothelial cells at 2 and 12 h of infection. Levels of activated Rho, captured using Rhotekin, in the cytoplasmic and membrane fractions were determined by Western blotting using anti-RhoA antibodies. In uninfected endothelial cells, activated Rho was observed primarily in the cytosolic fraction. At 2 h after infection, there was no substantial increase in the total amount of activated Rho compared to that of the control, but the activated Rho was translocated from the cytosol to the membrane fraction. At 12 h after infection there was an increased level of activated Rho, which was primarily located in the membrane fraction (Fig. 6E).

DISCUSSION

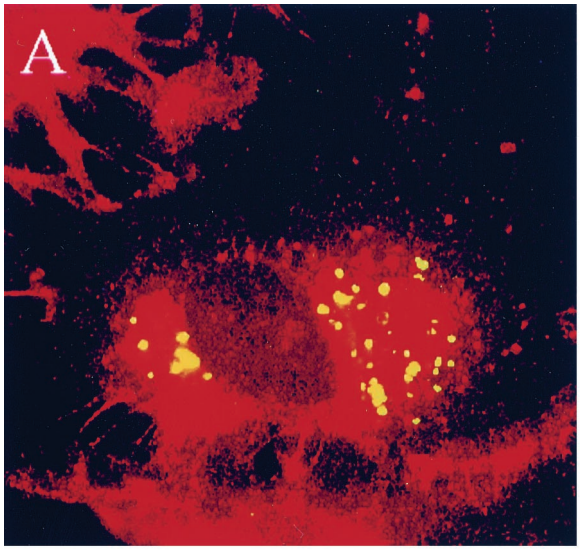
Pathogenic bacteria, especially intracellular pathogens, utilize host signaling and response mechanisms as an indispensable component of the infectious process. Many pathogenic bacteria facilitate their entry by inducing a rearrangement of the host cell actin cytoskeletal network, which is controlled in part by the intracellular signaling protein, Rho. The experiments discussed here demonstrate that infection of endothelial cells by *B. bacilliformis* is both dependent on Rho and results in activation of Rho.

Preincubation of endothelial cells with C3 exoenzyme almost entirely prevents entry of *B. bacilliformis*. The primary target of C3 exoenzyme is Rho, and as used in our protocols, we have directly demonstrated the inactivation of intracellular Rho by the C3 exoenzyme. This observation demonstrates that C3 exoenzyme-inactivatable proteins, Rho, or possibly other Rho family proteins are involved in entry and in fact are required for the process (Fig. 5B). C3 exoenzyme has only a low level of ADP ribosylation activity against Rac and Cdc42. It has been estimated that inactivation of Rho by C3 exoenzyme in vitro is at least 100-fold more efficient than that for Rac and 400-fold more efficient than that for Cdc42 (30). This low level of activity is believed not to be of physiological significance (19), so active Rho is almost certainly required for the initial infection.

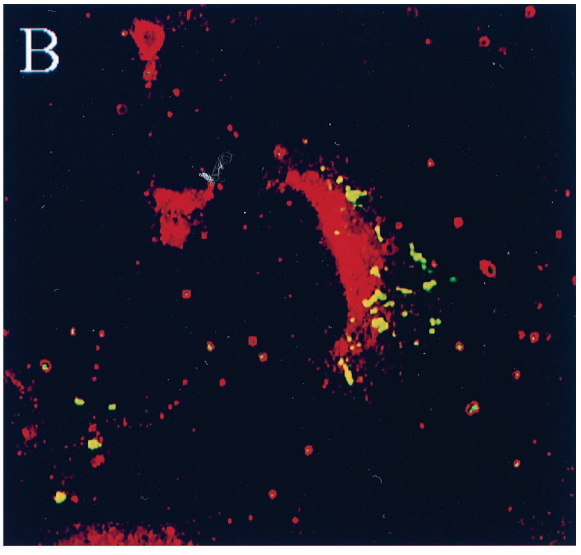
When C3 exoenzyme is added at various times after the bacteria, further internalization of the bacteria is prevented (Fig. 5C and D). In the absence of C3 exoenzyme, for up to at least 24 h, both the percentage of cells infected and the number of bacteria per cell (determined by mean cell fluorescence) continue to increase with time, but further increases in both these parameters can be prevented by the addition of C3 exoenzyme at any time (except that about 4 h is required for C3 exoenzyme to exert its effect). Corroborating this conclusion, incubation of C3 exoenzyme with endothelial cells, which had previously been infected with nonfluorescent *B. bacilliformis*, almost entirely prevents the subsequent entry of added, addi-

FIG. 3. Localization of fluorescent bacteria with the immunofluorescent staining of PECAM-1 and Golgi complex markers. Confluent monolayers of endothelial cells were infected at a multiplicity of 1:100 with *B. bacilliformis* expressing GFP for 24 h, fixed with 4% paraformaldehyde, and stained with anti-PECAM-1 antibodies, external membrane markers showing the perinuclear colocalization of fluorescent bacteria with PECAM-1 staining (A), anti-KDEL receptor antibody staining (B), anti-human Golgin-97 antibody staining (C), and BODIPY-TR ceramide staining (D), and then the nucleus was stained with DAPI (blue). Staining of the Golgi complex (red) with all three markers in infected endothelial cells showed colocalization of intracellular green fluorescent bacteria in the Golgi complex region. Bar, 10 μm. (E and F) Infected endothelial cells were incubated with BFA (1 μg/ml) for 15 min prior to infection, and BFA was present during the entire course of infection. (E) Infected endothelial cells showing a disrupted Golgi complex (red) and the dispersed green fluorescent bacteria in the cytoplasm. (F) Graph showing the mean fluorescence (indicating the internalized bacteria) of the infected cells incubated with or without BFA at the different time periods of infection. At 24 h of infection, a large decrease (>50%) was observed in the mean fluorescence, indicating the inhibition of internalization of the bacteria.

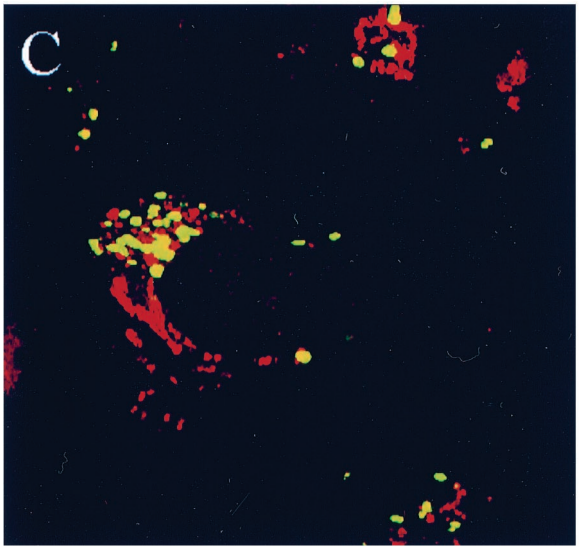
Anti-PECAM-1



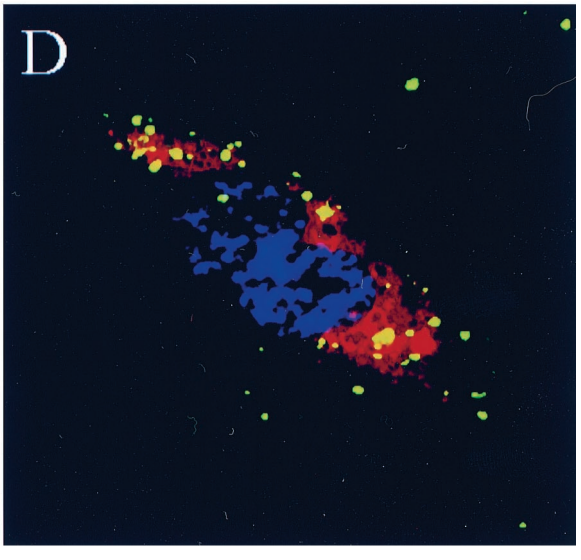
Anti-KDEL Receptor



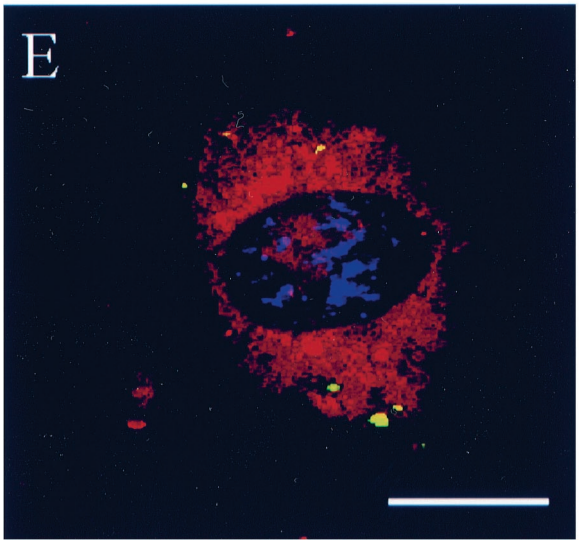
Anti-Human Golgin 97

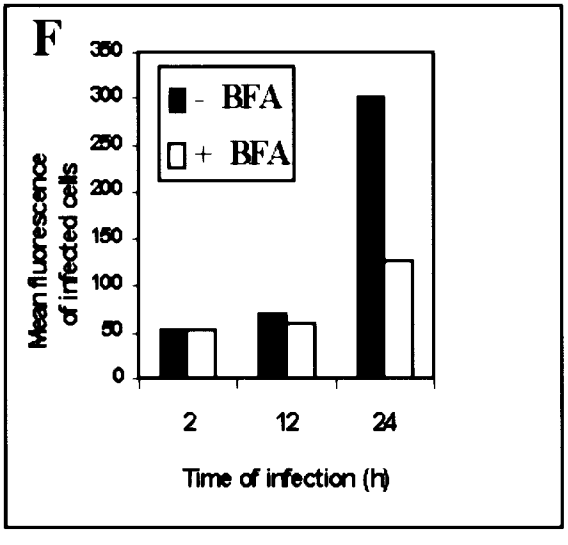


BODIPY-TR Ceramide



BFA + BODIPY-TR Ceramide





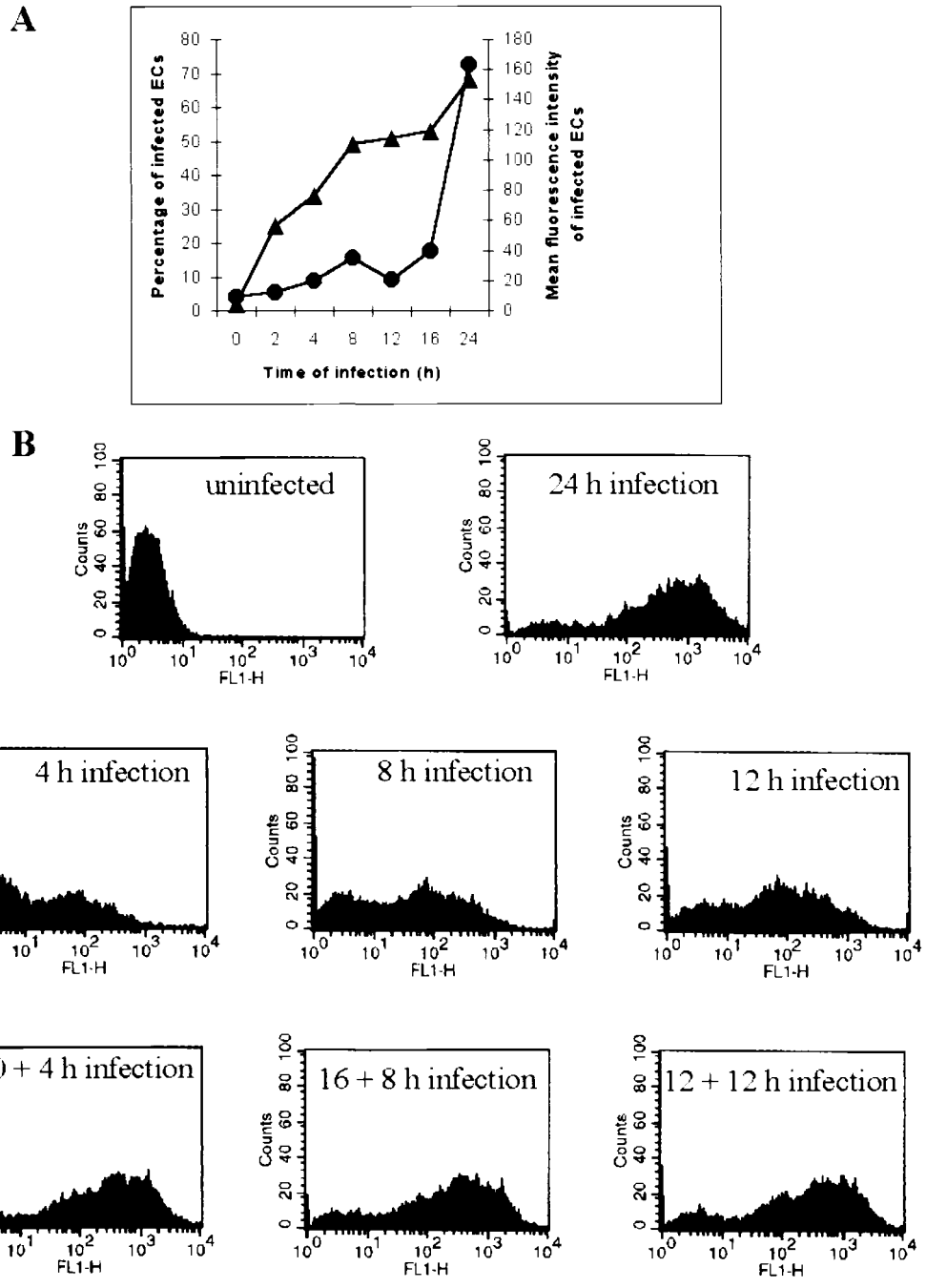


FIG. 4. Time course of endothelial cell infection with *B. bacilliformis*. Endothelial cells plated in gelatin-coated six-well culture plates were infected at a multiplicity of 1:100 with *B. bacilliformis* expressing GFP. Infected endothelial cells were trypsinized, and the time course of the infection was analyzed by the flow cytometric analysis described in Materials and Methods. (A) Percentage of fluorescent (infected) endothelial cells (ECs) (▲) and mean fluorescence intensity (●) of the infected endothelial cells at 0, 2, 4, 8, 12, 16, and 24 h of infection. (B) Endothelial cells were infected with nonfluorescent bacteria for 20, 16, or 12 h, followed by addition of fluorescent bacteria for 4, 8, and 12 h (to accomplish a total time of 24 h of infection), and were analyzed by flow cytometry. The uptake of the fluorescent bacteria after 24 h of infection in these endothelial cells was compared to that of their respective controls (endothelial cells infected with fluorescent bacteria only for 4, 8, and 12 h).

tional, fluorescent *B. bacilliformis* into the previously infected cells (data not shown).

In uninfected cells, initial entry of the bacteria might be dependent on the endogenous level of activated Rho. Levels of activated Rho might vary from cell to cell, which could explain the observation that 35% of the cells are not infected at all within 24 h, under conditions in which about 30% are infected within the first 2 h and the remaining 35% are progressively infected over the next 22 h (Fig. 4A). Alternatively, the extracellular bacteria may have the ability to activate intracellular Rho by some mechanism, such as interaction directly with specific target receptors or by transfer of proteins directly to

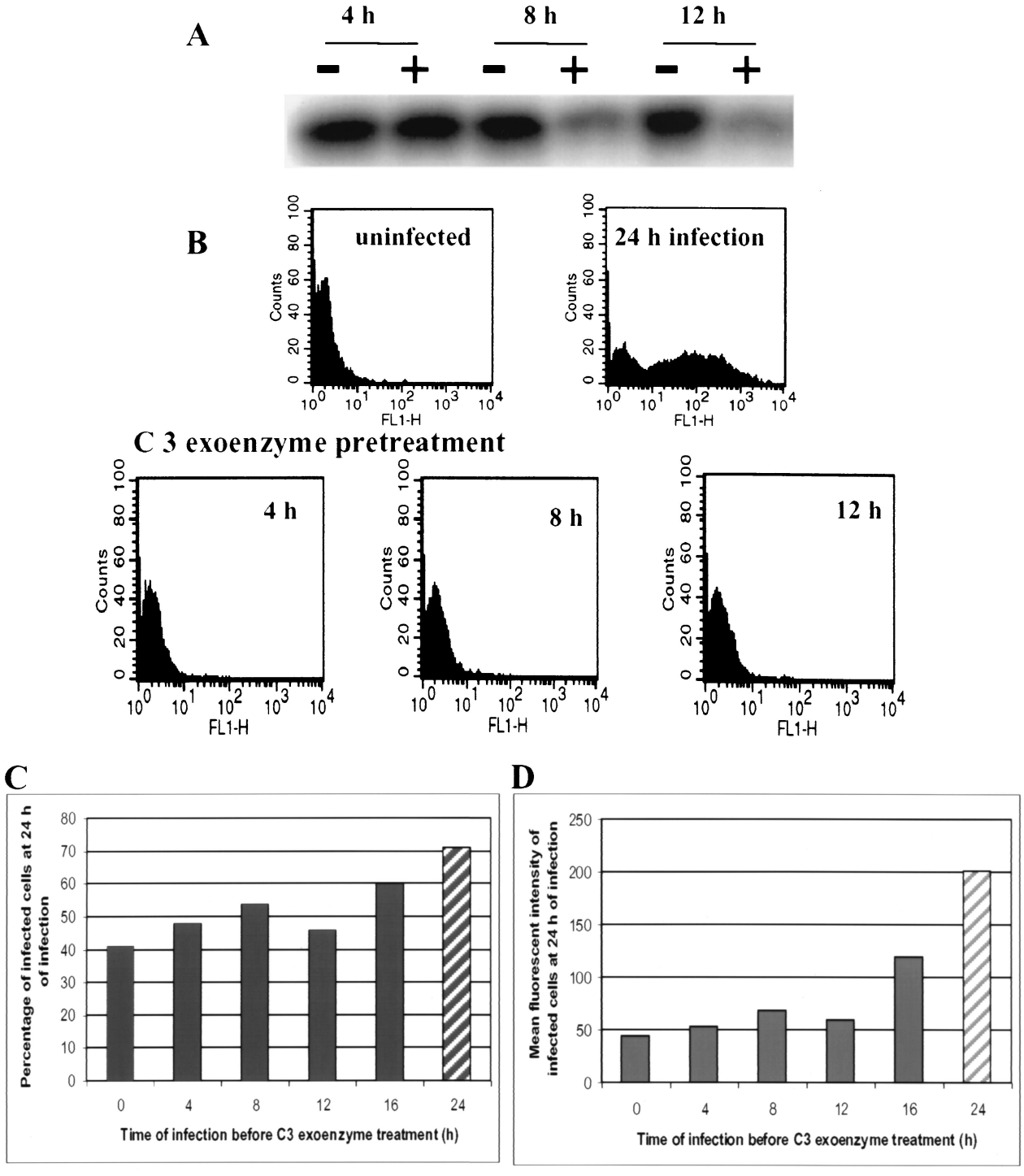


FIG. 5. C3 exoenzyme pretreatment and posttreatment of endothelial cells inhibit the internalization of *B. bacilliformis*. (A) Confluent monolayers of the endothelial cells cultured on gelatin-coated tissue culture plates were incubated with 100 μg of C3 exoenzyme/ml (+) or without C3 exoenzyme (−) for 4, 8, or 12 h. Treated and untreated endothelial cell lysates were [^{32}P]ADP ribosylated in vitro. A decrease in the signal intensity of the in vitro ADP-ribosylated Rho in treated endothelial cells indicates the increased in vivo ADP ribosylation by added C3 exoenzyme. (B) Flow cytometric plots of endothelial cells preincubated with 100 μg of C3 exoenzyme/ml for 4, 8, or 12 h and then infected with *B. bacilliformis* for an additional 24 h, and then cells were compared to uninfected and 24-h-infected endothelial cells without any C3 exoenzyme incubation. C3 exoenzyme was added to the infected endothelial cells at different times, i.e., at 0 h (C3 exoenzyme added simultaneously with the bacteria) or at 4, 8, 12, or 16 h after the infection with *B. bacilliformis* and were analyzed by flow cytometry for the percentage of the infected cells (C and D) and mean fluorescent intensity (C). (D) Both parameters were compared with those of the 24-h-infected endothelial cells (hatched bar) without any C3 exoenzyme incubation.

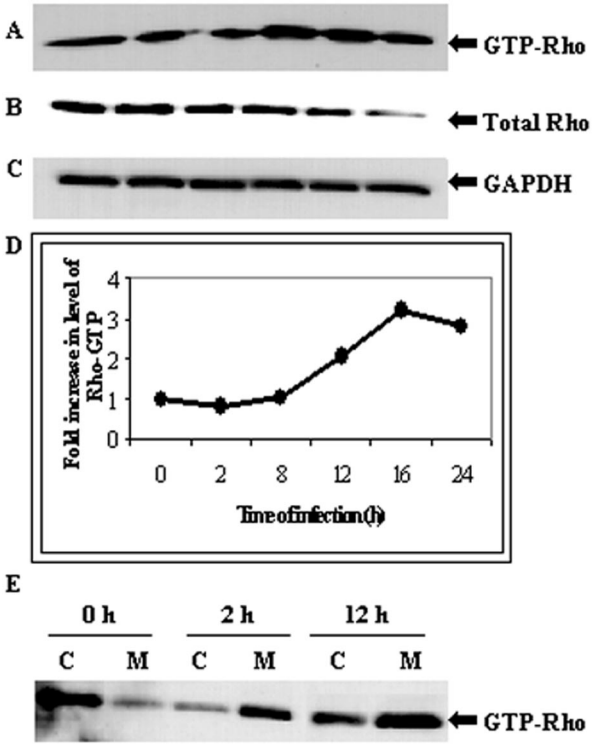


FIG. 6. Activated Rho (GTP-bound form) at different times of infection and its distribution in the cytosolic and membrane fractions. Equal amounts of endothelial cell lysates from the uninfected (0 h) and infected (2, 8, 12, 16, or 24 h) endothelial cells were analyzed by Western blotting with anti-RhoA or anti-GAPDH antibodies as described in Materials and Methods. (A) GTP-bound form of Rho captured with GST-RBD affinity beads and visualized with anti-RhoA antibodies by using the ECL Western detection kit. (B) Total amount of Rho in the endothelial cell lysates. (C) Endothelial cell lysates probed with anti-GAPDH antibodies (same blot as in panel B, after stripping). (D) Relative increase in activated Rho after infection (densitometric quantification of A). (E) Distribution of activated Rho between cytoplasmic (C) and membrane (M) fractions in uninfected and infected endothelial cells at 2 and 12 h of infection.

the host cell cytoplasm through the Type III protein export system. It is known that *B. bacilliformis* contains one or more genes related to ATP-dependent transporters (*txpA*, GenBank accession no. U68242, Upeslcis and Ihler, 1996; Derrick and Ihler, unpublished data).

With time, Rho becomes progressively activated in endothelial cells between 12 and 24 h of infection and is maximally activated at 16 h (Fig. 6A). Since infected and uninfected cells could not be separated in these experiments, it remains an open question whether activation of Rho occurs largely or exclusively in the infected cells or is induced by extracellular bacteria in all cells, including the uninfected cells.

Intracellular bacteria can be seen very soon after addition of bacteria to the endothelial cells, but a marked increase was observed in the number of bacteria per infected cell between 16 and 24 h (Fig. 4A), at the time when Rho was maximally activated, signifying a greatly increased rate of internalization of *B. bacilliformis*. This marked increase in mean cell fluorescence (about fourfold) over a short time seems to us to be too large to be due simply to intracellular growth of the bacteria (which have an extracellular doubling time of 8 to 24 h, depending on conditions). The addition of fluorescent bacteria at various times after an initial incubation with nonfluorescent bacteria directly demonstrates that internalization is a continuous process and also that an increased internalization of bacteria occurs at 16 to 24 h (Fig. 4A). Moreover, as with bacterial entry at any other time, the increased internalization can be prevented by prior addition of C3 exoenzyme. We conclude that the increased internalization seen after 16 h is due to the prior, maximal activation of Rho. *Shigella flexneri* is another bacteria known to involve Rho in process of internalization in the eukaryotic cells, which can be blocked by C3 exoenzyme treatment (1, 35).

Within 2 h after addition of the bacteria, much of the activated Rho had translocated from the cytosol to membrane fractions and both activation and translocation became much more extensive by 12 h. Rho-GTPase activated by *B. bacilliformis* infection binds to Rhotekin and so is in the GTP-bound form and is functional with respect to its ability to relocate to membranes. Rho-GTPases are involved in bacterial internalization by actin-dependent phagocytosis (7), either by causing plasma membrane protrusions to engulf the bacteria (Rac and Cdc42 dependent) or by actin-lined invaginations in the membrane whose formation is Rho dependent. The results of Dehio et al. (10) have shown that the leading lamella of the plasma membrane is intimately involved in internalization of *B. henselae*, which suggest the involvement of Rac and Cdc42. Cytotoxic necrotizing factor from *E. coli* is known to activate not only Rho but also Rac and Cdc42 (22). Experiments are underway to determine if activation of Rac and Cdc42 is involved in internalization of *B. bacilliformis*.

The visible morphological changes in endothelial cells infected with *B. bacilliformis* include the appearance of internal vesicles (which apparently fuse over time to form larger vesicles) (data not shown). At later time, large numbers of bacteria, present singly or in clumps, can be seen intracellularly by fluorescence microscopy, mostly in the perinuclear area. Both perinuclear bacteria and those located more distantly in the cytoplasm colocalize with PECAM-1, a marker of the external membrane, as detected by anti-PECAM-1 antibody staining. This suggests that the bacteria have entered the cell within vacuoles formed at the external membrane and are transported within those vacuoles to a perinuclear destination containing external membrane markers.

The perinuclear bacteria were colocalized with the Golgi complex, as shown using two specific protein markers and a lipid marker for the Golgi complex. Possibly, the perinuclear location of internalized bacteria results from a passive dependence on host vesicular transport to the Golgi complex. Once at the Golgi, the bacteria could possibly modify host proteins within the Golgi complex or utilize the Golgi complex to distribute bacterial proteins. Disruption of the Golgi complex with BFA leads to the dispersion of the bacteria from their perinuclear location and to a more general distribution throughout the cytoplasm. Using flow cytometry, we observed a large decrease after 24 h (>50%) of internalized bacteria in endothelial cells incubated with BFA (Fig. 3F). Pretreatment of eukaryotic cells with BFA has been reported to cause a dose-dependent inhibition of *Yersinia* invasin-mediated cell entry, although cell invasion by *Salmonella* was not affected (16). Inhibition of internalization by BFA might or might not be closely related to the mechanism of uptake.

Activation of Rho with *Bordetella bronchiseptica* dermonecrotizing toxin is known to induce proliferation of cytoplasmic membrane organelles, including Golgi, and formation of caveolae (35). This suggests that the key property of Rho with respect to infection may be to increase the rate of internalization of plasma membrane and bacteria bound to the membrane. We conclude that entry of *B. bacilliformis* into endothelial cells is initially Rho dependent, remains Rho dependent

during the infection, and is facilitated by higher levels of activated Rho.

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